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Achikanu, Cosmas; Pendekanti, Venkata Ganesh; Teague, Rebecca; Publicover, Stephen

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Effects of pH manipulation, CatSper stimulation and Ca^{2+}-store mobilisation on $[Ca^{2+}]_i$ and behaviour of human sperm

Running title: pH, Ca^{2+} and regulation of human sperm behaviour

Cosmas Achikanu[1], Venkata Pendekanti, Rebecca Teague[2] and Stephen Publicover*

School of Biosciences, the University of Birmingham, Birmingham, B15 2TT, UK.

[1]Current address: Department of Applied Biochemistry, Faculty of Applied Natural Sciences, Enugu State University of Science and Technology, Ebeano City, PMB 01660, Enugu, Enugu State, Nigeria.

[2]Current address: Genome Damage and Stability Centre, Science Park Road, Falmer, Brighton, BN14JY, UK

***Correspondence address**: s.j.publicover@bham.ac.uk

Abstract.

Study question: How do the alkaline pH, progesterone and 4-aminopyridine interact in their effects on human sperm?

Summary answer: Behaviour of human sperm (proportion of hyperactivated cells and motility kinematics) were related directly to $[Ca^{2+}]_i$ irrespective of pH or the agonist applied.

What is known already? CatSper channels of human sperm, which are central to generation of sperm $[Ca^{2+}]_i$ signals and induction of hyperactivated motility, are activated by intracellular alkalinisation and progesterone. Progesterone (P4) is much less effective than 4-aminopyridine (4-AP) (which mobilises stored Ca^{2+} but also raises pH_i) as an inducer of hyperactivation.

Study design, size, duration: This was a laboratory study, spanning approximately 18 months that used 15 sperm donors and involved more than 100 separate experiments.

Participants/materials, setting, methods: Semen donors and patients were recruited in accordance with local ethics approval (ERN_12-0570R). $[Ca^{2+}]_i$ responses of suspended cell populations were examined by fluorimetric recording and motility parameters assessed by computer-assisted sperm analysis (CASA).

Main results and the role of chance: Increasing pH_o from 7.4 to 8.5 raised pH_i (from 6.9 to 7.2) and significantly increased both $[Ca^{2+}]_i$ and the proportion of hyperactivated cells. Stimulation of cells with P4 (1 nM-20 μM) induced a biphasic (transient and plateau) increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase was of similar amplitude and dose-dependency at pH_o=7.4 and pH_o= 8.5. 4-aminopyridine (0.2-5 mM) induced a biphasic $[Ca^{2+}]_i$ increase that was dose-dependent across the entire range tested and was strongly enhanced at pH 8.5. Motility was assessed 300 s post-stimulation, during the plateau phase of the progesterone and 4-AP-induced $[Ca^{2+}]_i$ responses. Progesterone had only a small effect on hyperactivated motility even at the highest dose used (20 μM; <5% increase in the proportion of cells classified as hyperactivated) which was insensitive to pH_o. 4-aminopyridine potently stimulated hyperactivated motility, this effect being dose-dependent and greatly enhanced at pH_o=8.5. The

relationship between $[Ca^{2+}]_i$ (fluorescence of fluo4) and proportion of hyperactivated cells, irrespective of pH_o, agonist or dose, was fitted by a single curve (2^{nd} order polynomial; R^2=0.96). Similar analysis of curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) showed a linear relationship to $[Ca^{2+}]_i$ (R^2>0.9).

Limitations, reasons for caution: This was an *in-vitro* study and caution must be taken when extrapolating these results to *in vivo* regulation of sperm. Though controls indicate that saturation of fluo4 did not affect the findings, at the highest doses of progesterone the true amplitude of the $[Ca^{2+}]_i$ transient may not have been reported by the dye.

Wider implications of the findings: These findings indicate that (i) activation of human sperm CatSper by progesterone (and presumably other ligands that act similarly) and consequent acquisition of hyperactivated motility is not significantly enhanced by intracellular alkalinisation; (ii) VCL, ALH and hyperactivation are directly related to $[Ca^{2+}]_i$, irrespective of the mechanism by which Ca^{2+} is mobilised, and the ability of stimuli to induce prolonged $[Ca^{2+}]_i$ elevation (as occurs upon mobilisation of stored Ca^{2+}) determines the observed effect on cell behaviour.

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Key words: spermatozoa/pH/progesterone/calcium/CatSper/hyperactivation

Introduction

Freshly ejaculated mammalian sperm swim with a low-amplitude, symmetrical flagellar beat that generates progressive movement, termed activated motility. Within the female tract (or when incubated under appropriate conditions *in vitro*) some cells become hyperactivated, a behaviour characterised by exaggerated (often asymmetric) bending of the flagellum which causes greatly increased lateral excursion of the sperm head and may result in continuous turning such that the cell fails to progress (Kay and Robertson, 1988; Suarez, 2008). Hyperactivation is essential for mammalian fertilisation. Cells that fail to hyperactivate cannot successfully ascend the female tract or penetrate the zona pellucida (Carlson, et al., 2003; Ho, et al., 2009).

The primary regulatory signal for the transition from activated to hyperactivated motility in sperm of humans (and most mammals where this has been investigated) is an increase in $[Ca^{2+}]_i$ (Ho, et al., 2002; Bedu-Addo, et al., 2008; Suarez, 2008). The sperm-specific, Ca^{2+}-permeable channel CatSper plays a central role in this process. Sperm of CatSper-null mice fail to hyperactivate and are consequently infertile, and loss of CatSper function in human sperm appears to have similar effects (Carlson, et al., 2003; Smith, et al., 2013; Williams, et al., 2015). CatSper channels are weakly voltage sensitive and are also activated by intracellular alkalinisation, which in mouse and bovine sperm may be the key regulator of the channel (Lishko, et al., 2012). Within the female tract both the alkaline environment (which varies both spatially and temporally) and sperm capacitation will increase pHi, regulating the sperm's behaviour through control of CatSper activity (Cross and Razy-Faulkner, 1997; Fraire-Zamora and Gonzalez-Martinez, 2004; Lishko et al., 2012; Nishigaki, et al., 2014; Ng, et al., 2018). In human (and other primate) sperm the regulation of CatSper appears to be more complex. Though human CatSper is pH sensitive, it is also activated by a wide range of agonists (Lishko, et al., 2011; Strunker, et al., 2011; Brenker, et al., 2012). The best characterised of these, progesterone (P4), occurs at high (micromolar) concentrations in follicular fluid and cumulus but may also be present throughout the tract at concentrations sufficient to regulate activity of the channel (Correia, et al., 2007). In electrophysiological studies stimulation by 500 nM P4 and alkaline pH interacted

synergistically (Lishko et al., 2011), but the effects of such interaction on $[Ca^{2+}]_i$ and hyperactivation have not been described.

In addition to CatSper-mediated Ca^{2+}-influx, regulation of sperm motility by $[Ca^{2+}]_i$ can occur through mobilisation of stored Ca^{2+}, probably from organelle(s) at the sperm neck/midpiece (Ho and Suarez, 2001; Bedu-Addo, et al., 2008). 4-aminopyridine (4-AP), a particularly potent inducer of hyperactivation (Gu, et al., 2004), is a weak base and will stimulate CatSper by raising pH_i, but it has also been shown to mobilise stored Ca^{2+} in a number of cell types including human sperm (Gobet, et al., 1995; Grimaldi, et al., 2001; Bhaskar, et al., 2008; Alasmari, et al., 2013b; Kasatkina, 2016). 4-AP induced hyperactivation in human sperm that were functionally CatSper null (Williams, et al., 2015). When we compared the effects of P4 and 4-AP on motility we found that, even at saturating concentrations, P4 was less effective than 4-AP as an inducer of hyperactivated motility, though it induced a significantly larger $[Ca^{2+}]_i$ response (Alasmari, et al., 2013b).

Though it is established that diverse stimuli induce hyperactivation of human sperm via elevation of $[Ca^{2+}]_i$, we know little of how such stimuli interact and combine in their effects on motility. Are the actions of these stimuli simply integrated by $[Ca^{2+}]_i$ irrespective of their origin, or does the nature of the original stimulus and/or the source of Ca^{2+} affect the strength of the response? What is the relationship between $[Ca^{2+}]_i$ and hyperactivation and do components of hyperactivated motility (increased flagellar excursion causing enhanced lateral head movement, asymmetric beating causing turning/path curvature) show similar $[Ca^{2+}]_i$ sensitivity? We have used fluorimetric assay of $[Ca^{2+}]_i$ and computer-assisted sperm analysis (CASA) to investigate the interacting effects of P4, 4-AP, and elevated pH_i, on $[Ca^{2+}]_i$ and hyperactivation in human sperm.

Materials and Methods

Materials, salines

Details of materials and salines (supplemented Earle's balanced salt solution; sEBSS) are provided in Supplementary Information Materials and Methods.

Ethical approval

Written consent was obtained from donors in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (University of Birmingham ERC 07-009 and ERN-12-0570).

Selection and preparation of spermatozoa Semen samples were from donors with normal sperm concentration and motility (WHO 2010). Samples were obtained by masturbation after 2-3 days sexual abstinence. After liquefaction (30 min), sperm were swum up into sEBSS (60 min), adjusted to ≈6 million/ml and left to capacitate (37°C, 6% CO_2) for 5 hours (Alasmari et al., 2013b).

Assessment of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was assessed in fluo4-loaded cells using a FLUOstar microplate reader (BMG Labtech Offenburg, Germany). Details of methodology are provided in Supplementary Information Materials and Methods. Parallel controls with dimethyl sulfoxide (DMSO (vehicle)) at a concentration equivalent to that present in the highest dose used (1% for 4-AP; 0.2% for P4) showed small, inconsistent effects on fluorescence (Supplementary Information Figure. S1a, b). At lower doses (0.00001-0.1%) no effects were detected.

Assessment of pH_i

pH_i was assessed in 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein,, acetoxymethyl ester (BCECF, AM)-loaded cells using a FLUOstar microplate reader (BMG Labtech Offenburg, Germany). Details of methodology, measurement and calibration are provided in Supplementary Information Materials and Methods.

Assessment of motility

Motility of sperm samples was assessed at 35°C using a Hamilton Thorn CEROS CASA system. Details of methodology are provided in Supplementary Information Materials and Methods.

Statistical analysis

Statistical significance was determined using Student's paired/unpaired t-test or analysis of variance (ANOVA) and adjusted using the Holm-Bonferroni correction for multiple comparisons (Gaetano, 2013) as appropriate. Percentage data were transformed using the arcsine square root conversion (Sokal and Rohlf, 1981) before statistical analysis to allow application of parametric tests. Data are presented as mean ± SEM. Unless stated otherwise, the values of 'n' for $[Ca^{2+}]_i$ and motility assessments provided in text and figure legends show the number of experiments used for statistical analysis.

Results

pH_o and pH_i.

To assess the effect of manipulating extracellular pH on pHi, BCECF-loaded sperm were suspended in media buffered to a range of pH values (6.0-9.0) then exposed to 0.12% Triton-X-100 (Fraire-Zamora and Gonzalez-Martinez, 2004), allowing BCECF ratios to be recorded both from intact cells and after permeabilsation to equilibrate pHi with pH_o (Supplementary Information Figure S2a). Using a calibration curve for BCECF fluorescence obtained from permeabilised cells (Supplementary Information Figure S2b) we estimated pH_i for intact cells. As described previously (Hamamah, et al., 1996), pH_i was strongly correlated with pH_o (Supplementary Information Figure. S2c). To investigate the effects of pH on $[Ca^{2+}]_i$ signalling and motility in human sperm we selected values for pH_o of 7.4 and 8.5 (pH_i=6.85±0.06 and 7.19±0.14 respectively; n=5 experiments; P=0.02; figure 1a). The proportion of motile cells was slightly greater at pH_o=8.5 (figure 1b; $p<0.05$) and the proportion of progressively motile was not affected (figure 1c). In contrast, both resting $[Ca^{2+}]_i$ and hyperactivated motility (% hyperactivated cells) were markedly increased at pH_o=8.5 compared to pH_o=7.4 (figure 1d,e; $P<10^{-5}$ and $P<10^{-6}$ respectively).

Interaction of progesterone and pH.

When P4 was applied to cells suspended in standard sEBSS (pH_o=7.4) we observed an increase in $[Ca^{2+}]_i$ that peaked within 20 s, decayed over the following 60-90 s and was followed by a plateau phase which was maintained for the duration of recording (300 s; figure 2a left panel). Both transient and plateau saturated at 0.1-1 μM P4 and were clearly detectable (and statistically significant compared to the preceding control period) when the cells were stimulated with doses as low as 1 nM (figure 2b, c; grey bars). In parallel experiments carried out with cells that had been suspended in saline buffered to pH 8.5, P4 induced a similar biphasic $[Ca^{2+}]_i$ elevation though the decay of the transient was clearly slower under these conditions (figure 2a right panel). P4-induced transient and plateau responses were both dose dependent ($P=1.3*10^{-9}$ and $2.7*10^{-6}$ for transient and plateau

respectively) but there was no significant effect of pH_o (P=0.12 and 0.83 for transient and plateau respectively, 2-way ANOVA; figure 2b, c). Under both conditions the transient and plateau responses to P4 (20 μM) significantly exceeded those seen in parallel controls where vehicle (DMSO) was applied at equivalent concentration (figure 2b, c)

The failure of elevated pH to enhance the increment in fluorescence induced by P4 concentrations ≥0.1 μM might reflect limitations of the assay. If saturation of fluo4 occurs during the $[Ca^{2+}]_i$ transient the dye would fail to report any enhancement of the $[Ca^{2+}]_i$ signal at pH_o=8.5. To investigate this we carried out further experiments in which cells, suspended at pH_o=8.5, were first stimulated with P4 (0.001-20 μM) then 10 μM ionomycin was added 30-40 s after the peak of the P4-induced $[Ca^{2+}]_i$ transient. In 6 experiments ionomycin consistently increased fluorescence above the levels induced by P4, the size of the maximum fluorescence increase being 40-50% greater than that reached during the $[Ca^{2+}]_i$ transient induced by saturating concentrations of P4 (P<0.01;Supplementary Information figure S3a, b). Though this suggests that our failure to observe an enhanced $[Ca^{2+}]_i$ response to P4 at pH_o=8.5 was not due to dye saturation, we further investigated this by assessing responses to 0.1 μM and 1 μM P4 using a lower affinity dye (Fluo5F), which has a reported *in vitro* K_d of 2.35 μM compared to 345 nM for fluo4 (6.8-fold difference; Gee et al., 2000). There was now a clear difference in the amplitude of the P4-induced transient at both doses of P4 (P<0.005; Supplementary Information figure S3d), which was not apparent when using fluo4. However, we still observed no significant enhancement of the P4-induced $[Ca^{2+}]_i$ signal at pH_o= 8.5 (P>0.5; Supplementary Information figure. S3c,d).

We next examined the effect of pH_o on the ability of P4 to induce hyperactivated motility. Stimulation of sperm suspended in standard sEBSS with P4 (0.1-20 μM) induced a small (<5%) but significant increase in the proportion of hyperactivated cells that was dose-independent over the range used (figure 2d; grey bars). In parallel experiments with cells suspended at pH_o=8.5 the effect was of similar amplitude (figure 2d; black bars). Analysis of the data by 2-way ANOVA confirmed that neither the effect of P4 dose nor the effect of pH_o was significant (P=0.72 and 0.89 respectively).

Interaction of 4-AP and pH.

Similarly to the effect of P4, application of 4-AP to cells suspended in standard sEBSS (pH_o=7.4) induced a biphasic increase in $[Ca^{2+}]_i$ (Alasmari et al, 2013a). The initial transient reached a maximum after 20-30 s, decayed over the following 50-60 s and was then followed by a plateau which was maintained for the duration of recording (300 s; figure 3a left panel). The amplitudes of both transient and plateau (300 s post-stimulation) were dose-dependent ($P=6*10^{-6}$ and $P=2.*10^{-5}$ respectively; 1-way ANOVA). $[Ca^{2+}]_i$ elevation was clearly detectable at the lowest concentration of 4-AP tested (0.2 mM; $P<0.01$ compared to preceding control period), but this effect apparently saturated at 0.4-0.6 mM, a further enhancement occurring at concentrations >1 mM (figure 3b,c grey bars). Both transient and plateau responses to 4-AP (5 mM) significantly exceeded those seen in parallel controls where vehicle (DMSO) was applied at equivalent concentration (figure 3b,c; grey bars).

Application of 4-AP to cells suspended at pH_o=8.5 induced dose-dependent $[Ca^{2+}]_i$ responses similar to those seen at pH 7.4 though the amplitudes of both transient and sustained responses were significantly greater ($P=6.9*10^{-4}$ and $P=1.9*10^{-6}$ respectively; 2-way ANOVA; figure 3). As with responses to P4, decay of the $[Ca^{2+}]_i$ transient was slower at the higher pH_o (figure 3a). Whereas the sustained response induced by P4 was relatively small and of consistent amplitude relative to the preceding transient (25-30% at 300 s post-stimulation; figure 3e; yellow symbols), in 4-AP-stimulated cells the sustained response became larger (relative to the preceding transient) as the dose was increased (figure. 3b, c; figure 3e; black symbols). When cells suspended at pH 8.5 were stimulated with the highest dose of 4-AP (5 mM) we observed no decay of the $[Ca^{2+}]_i$ signal after the initial peak (figure 3a; right panel).

Stimulation with 4-AP of cells suspended in standard sEBSS (pH 7.4) had little effect on motility at doses of 0.2-0.6 mM but with concentrations of 4-AP ≥0.8 mM we observed a significant and dose-dependent increase in the proportion of hyperactivated cells (figure 3d; $P<0.05$). When cells were suspended at pH_o=8.5 significant stimulation of hyperactivated motility was seen at all doses tested

(0.2-5 mM) and the effect was strongly dose dependent (figure 3d). Analysis of the data by 2-way ANOVA confirmed that both dose-dependence and pH_o sensitivity were highly significant ($P=1.7*10^{-12}$ and $1.5*10^{-21}$ respectively). Motility (% cells) was not affected by exposure to 5 mM 4-AP for 300 s, either at $pH_o=7.4$ or $pH_o=8.5$ ($P>0.3$).

Is the effect of 4-AP due to cytoplasmic alkalinisation?

4-AP is much more potent than P4 in stimulating hyperactivation of human sperm (demonstrated in this study see figures 2d, 3d and our previous studies Alasmari et al, 2013a,b). Previously we established that, in cells suspended in standard sEBSS (pH 7.4) this effect was not due merely to the ability of 4-AP (a weak base) to raise pH_i, since 25 mM NH_4Cl, which had a similar effect on cytoplasmic pH_i, had only modest effects on motility (Alasmari, et al., 2013b). During this study we carried out similar experiments on cells suspended in saline buffered to pH 8.5. Exposure of cells to 2 mM 4-AP for 300 s increased pHi by ≈0.5 units and in parallel experiments the effect of 25 mM NH_4Cl on pH_i was greater (this difference was NS; Supplementary Information. figure. S4a). In contrast, the effect of 2 mM 4-AP on the proportion of hyperactivated cells was significantly greater than that of NH_4Cl, which had negligible effects on motility (Supplementary Information figure S4b).

Relationship between $[Ca^{2+}]_i$ and motility in sperm stimulated with P4, 4-AP and high pHo.

Stimulation of human sperm with P4 or by cytoplasmic alkalinisation elevates $[Ca^{2+}]_i$ and modifies motility primarily by activation of CatSper (Lishko, et al., 2012) whereas the more potent effects of 4-AP involve mobilisation of stored Ca^{2+} in addition to alkalinisation (see introduction). To compare the relative efficacies (regulation of sperm behaviour) of the $[Ca^{2+}]$ signals induced by P4 and 4-AP (and the effects on these of pH) we plotted, for each stimulus protocol (agonist concentration and pH), the mean fluo4 fluorescence intensity at 300 s post stimulation (time of CASA data collection) and mean values for CASA motility parameters (hyperactivation, VCL, ALH and LIN). Figure 4a shows the relationship between fluorescence intensity and % hyperactivated cells for four concentrations of P4 (0.1-20 μM; yellow symbols; controls shown green) and seven concentrations of 4-AP (0.2-5 mM; grey symbols; controls shown black). Experiments at $pH_o=7.4$ and 8.5 are plotted as circles and

250 triangles respectively. Though the cells used for the P4 experiments showed slightly higher levels of
251 'spontaneous' hyperactivation than those used during 4-AP experiments (compare green and black
252 control symbols), the points describe a single curve best fitted by a 2nd order polynomial ($y = 0.009x^2$-
253 $0.57x+8.6$; $R^2=0.96$; fig. 4a). Figure 4 panels b, c and d show equivalent plots for VCL, ALH and
254 LIN, the three kinematic parameters used to define hyperactivation in CASA analysis. Both VCL
255 (figure 4b) and ALH (figure 4c) were linearly related to fluo4 fluorescence ($R^2=0.93$ and 0.91
256 respectively). The relationship between fluo4 fluorescence and LIN was more complex. The data from
257 4-AP experiments fell on a single curve (2nd order polynomial; $R^2=0.97$) but, in cells stimulated with
258 P4, values for LIN were markedly lower (figure. 4d yellow symbols). LIN is calculated from the ratio
259 of VSL:VCL (blue:black lines in figure 5b) so this effect of P4 could reflect increased lateral
260 deviation of the sperm head (ALH) and/or greater curvature of the average path (red line in figure 5b).
261 Since ALH values from the P4 and 4-AP experiments clearly lie on the same line (figure 4c), this
262 suggests that P4 particularly increases path curvature. We therefore assessed straightness (STR; the
263 ratio VSL:VAP; blue:red lines in figure 5b), which is determined primarily by path curvature. Low
264 doses of P4 (0.1-1 μM) significantly reduce STR, similarly to their effect on LIN (compare figures 4d
265 and 5a). This effect was particularly marked at $pH_o=8.5$ (figure 5c). 4-AP had an equivalent effect on
266 STR only at the highest doses (1-5 mM; figure 5a, d), when $[Ca^{2+}]_i$ reached levels exceeding those
267 seen with P4 stimulation. Thus, at equivalent $[Ca^{2+}]_i$, P4 increased path curvature more than 4-AP.

268

Discussion

Mammalian sperm ascending the female tract experience an increase in pH from ≈4 in the vagina to ≈8.0 in the oviduct (Ng, et al., 2018). As reported previously (Hamamah *et al*., 1996), pH_i of human sperm was sensitive to pH_o. Surprisingly, though both $[Ca^{2+}]_i$ and hyperactivated motility were significantly enhanced by increased pH_o, consistent with activation of CatSper by alkalinisation, the effects of P4 (1 nM-20 μM) on $[Ca^{2+}]_i$ and motility showed negligible pH-sensitivity. Fraire-Zamora and Gonzalez-Martinez (2004) reported a similar lack of effect when using NH_4Cl to alkalinise the cytoplasm. Control experiments indicated that saturation of fluo4 did not 'hide' enhanced $[Ca^{2+}]_i$ transients and, more importantly, during the P4-induced $[Ca^{2+}]_i$ plateau recorded at pH_o=8.5 fluo4 was no more than 65% saturated, yet the P4-induced $[Ca^{2+}]_i$-increment was similar at pH_o=7.4 and 8.5. We conclude that increased pH_o and consequent cytoplasmic alkalinisation does not significantly enhance Ca^{2+}-mediated modulation of motility by P4. Thus, though the more alkaline pH of the upper female tract will modulate CatSper, increasing $[Ca^{2+}]_i$ and changing the behaviour of the sperm, sensitivity to the high concentrations of P4 encountered in the vicinity of the cumulus-oocyte-complex may be unchanged.

Similar experiments with 4-AP showed significant dose- and pH-dependent effects on both $[Ca^{2+}]_i$ and motility. At pH_o=7.4 we observed a small though significant effect on $[Ca^{2+}]_i$ which saturated at 0.2-0.6 mM and at higher doses there was a second and much greater effect on both $[Ca^{2+}]_i$ and hyperactivation. This complex effect is not surprising since 4-AP may both activate CatSper by raising pH_i and mobilise stored Ca^{2+} at doses as low as 0.25 mM (Alasmari et al, 2013b; Kasatkina; 2016). Chavez, et al. (2018) have recently shown that alkalinisation of the acrosome by weak bases (though 4-AP was not tested) induces release of stored Ca^{2+}. At pH_o=8.5 4-AP was effective even at low doses, probably because alkaline pH favours the non-ionised (cell-permeant) form of 4-AP, facilitating intracellular accumulation of the drug (Howe and Ritchie, 1991).

P4-induced hyperactivation of human sperm *in vitro* has been reported on a number of occasions and is clearly illustrated in the videos of Smith, et al. (2013) and Schiffer, et al (2014), yet in this study

295 (and others; e.g. Alasmari et al, 2013a,b) the potency of P4 compared to 4-AP was negligible. This
296 inconsistency may reflect the kinetics of the $[Ca^{2+}]_i$ responses in these *in vitro* experiments, where
297 agonists are applied as a bolus. P4-induced $[Ca^{2+}]_i$ transients are large but decay rapidly such that
298 when motility is assessed 300 s post-stimulus the effect of P4 is negligible. In contrast, the 4-AP-
299 induced signal decays little, particularly at high doses, so that persistent and potent stimulation of
300 hyperactivation is observed. We hypothesise that this difference reflects 4-AP-induced Ca^{2+} store
301 mobilisation (see above) and consequent activation of store-operated channels (Lefievre, et al., 2012).
302 In a minority of P4-stimulated cells CatSper-mediated Ca^{2+}-influx induces $[Ca^{2+}]_i$ oscillations
303 superimposed on the P4-induced $[Ca^{2+}]_i$-plateau, an effect which apparently reflects secondary release
304 of stored Ca^{2+} (Harper et al, 2004). Such $[Ca^{2+}]_i$-oscillations may be required for motility regulation
305 (Bedu-Addo et al., 2008) and may underlie the repeated switching between activated and
306 hyperactivated behaviours seen in human sperm (Publicover, 2017).

307 To investigate the relationship between pH, agonist type/concentration, $[Ca^{2+}]_i$, and sperm behaviour
308 we calculated mean $[Ca^{2+}]_i$ (absolute fluorescence intensity) and CASA kinematics for each condition
309 for which equivalent fluorimetric and CASA data had been collected (22 in total). Plotting of the
310 relationship between $[Ca^{2+}]_i$ and hyperactivation produced a curve that was best fitted by a 2^{nd} order
311 polynomial (R^2=0.96). Data fell on this curve irrespective of pH_o or agonist type. Similar analysis of
312 VCL and ALH (two of the kinematics used to define hyperactivation) generated linear plots
313 (R^2>0.91). These data clearly suggest that both curvilinear velocity and lateral movement of the sperm
314 head are determined primarily by $[Ca^{2+}]_i$, irrespective of pH or the nature of the agonist. The values of
315 VCL and ALH used to define hyperactivation (150 $\mu m.s^{-1}$ and 7 μM respectively; Mortimer, 2000)
316 both occurred at a fluorescence intensity of ≈90,000 (see figure 4; red dashed lines) . Data for LIN
317 from 4-AP experiments (but not P4, see below) were fitted by a 2^{nd} order polynomial, the
318 hyperactivation threshold value of 50% again occurring at ≈90,000. To obtain a rough estimate of
319 $[Ca^{2+}]_i$ we used the data from Harper, et al. (2003), who calibrated their ratiometric fura2 recordings
320 from human P4-stimulated sperm populations prepared and treated (apart from recording at 37^oC)
321 exactly as described here. Using the P4-induced $[Ca^{2+}]_i$ transient as a bioassay (Supplementary

Information figure S5), we estimate that $[Ca^{2+}]_i$ corresponding to a fluorescence of ≈90,000 (hyperactivation 'threshold' values for VCL/ALH/LIN) is 600-700 nM and that 50% hyperactivation (50% of cells satisfy all three kinematic criteria) occurs at ≈800 nM. In re-activated, 'skinned' bovine sperm a $[Ca^{2+}]$ of ≈200 nM induced 50% hyperactivation and the effect saturated at 400 nM (Ho et al., 2002).

Plotting of values for LIN and STR (straightness, which assesses the curvature of the sperm's average path) against $[Ca^{2+}]_i$ showed striking differences between cells stimulated with P4 and with 4-AP. At equivalent levels of fluo4 fluorescence, whereas LIN and STR were markedly reduced in P4-stimulated cells, 4-AP had no effect (in fact a non-significant increase in STR was recorded. Control values of LIN and STR in P4 experiments were lower than those in experiments with 4-AP (particularly at pH_o=7.4) and this observation should therefore be interpreted cautiously. However, this striking difference in the effects of P4 and 4-AP is not apparent in the data for VCL or ALH. Furthermore, at pH_o=8.5 P4 significantly decreased STR yet doses of 4-AP with equivalent effects on fluo4 fluorescence had no effect. One possibility is that P4 has 'extra', non-$[Ca^{2+}]_i$-dependent effects on curvature of the cell path. For instance, activation of Erk1⁄2, p90RSK, p38MAPK by P4 (Sagare-Patil, et al., 2012) may affect motility. This unexpected effect of P4 on motility deserves further investigation since induction of turning without other characteristics of hyperactivated motility may play a role in enabling sperm to locate the oocyte.

In summary, our data indicate a clear relationship between $[Ca^{2+}]_i$ and hyperactivation that is independent of pH or the mechanism of agonist-induced Ca^{2+}-mobilisation. 4-AP, which mobilises stored Ca^{2+}, is more effective in promoting persistent elevation of $[Ca^{2+}]_i$ and consequent modulation of sperm behaviour and Ca^{2+}-store-dependent mechanisms may, therefore, be important in tonic regulation of motility *in vivo*.

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Figure legends

Figure 1.

Manipulation of pH_i. **a:** mean pH_i at the values of pH_o used in this study. Each bar shows mean ± sem of 5 experiments (P=0.02; paired t test). **b** and **c** show proportion of motile cells (b) and progressively motile cells (c) at pH_o=7.4 and pH_o=8.5. Bars show mean ± sem of 34 experiments. **d:** Resting $[Ca^{2+}]_i$ (fluo4 fluorescence) at pH_o=7.4 and pH_o=8.5. Each bar shows mean ± sem of 14 experiments $P=1.2*10^{-6}$; paired t test). **e:** Proportion of hyperactivated cells at pH_o=7.4 and pH_o=8.5. Each bar shows mean ± sem of 34 experiments ($P=1.1*10^{-7}$; paired t test). P values shown above bars indicate comparison of pH_o=7.4 and pH_o=8.5.

Figure 2.

Interaction of P4 and pH. **a:** Effect of P4 on $[Ca^{2+}]_i$ (fluorescence of fluo4) at pH_o=7.4 (left panel) and pH_o=8.5 (right panel). Traces show mean response (n=8 experiments) to 20 μM (dark blue), 10 μM, 1 μM, 0.1 μM, 0.01 μM, 0.001 μM (dark green) P4. Arrows show time of P4 application. **b and c;** Dose-dependence of the fluorescence increment induced by P4 at the transient peak (b) and plateau (300 s post-stimulation; c) at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars); mean ± sem of 8 experiments. **d:** P4-induced increment in hyperactivation (difference in % hyperactivated cells compared to parallel untreated control) at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars); mean ± sem of 21 experiments. Asterisks indicate comparison between 20 μM P4 and equivalent DMSO controls (b, c) and between all P4 doses and untreated controls (d); P<0.05 (*), P<0.005 (***), P<0.001 (****), P<0.0005 (*****).

Figure 3.

Interaction of 4-AP and pH. **a:** Effect of 4-AP on $[Ca^{2+}]_i$ (fluorescence of fluo4) at pH_o=7.4 (left panel) and pH_o=8.5 (right panel). Traces show mean response (n=6 experiments) to 5 mM (dark blue), 2 mM, 1 mM, 0.8 mM, 0.6 mM, 0.4 mM and 0.2 mM (dark green) 4-AP. Arrows show time of 4-AP application. **b and c;** Dose-dependence of the fluorescence increment induced by 4-AP at the transient peak (b) and plateau (300 s post-stimulation; c) at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars); mean ± sem of 6 experiments. **d:** Dose dependence of 4-AP-induced increment in hyperactivation (difference in % hyperactivated cells compared to parallel untreated control) at 300 s post-stimulus at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars), mean ± sem of 13 experiments. Asterisks indicate comparison between 5 mM 4-AP and equivalent DMSO controls (b, c) and between all 4-AP doses and untreated controls (d); P<0.05 (*), P<0.01 (**), P<0.005 (***), P<0.001 (****), P<0.0005 (*****). **e**: Relationship between amplitude (increment in fluo4 fluorescence) of the $[Ca^{2+}]_i$ transient (x-axis) and sustained $[Ca^{2+}]_i$ signal (300 s post-stimulation; y-axis). Circles indicate experiments carried out at pH_o=7.4 and triangles indicate experiments carried out at pH_o=8.5. Yellow symbols show stimulation with P4 and grey symbols show stimulation with 4-AP. Each point shows mean of 8 experiments for P4 and 6 experiments for 4-AP.

Figure 4.

Relationship between $[Ca^{2+}]_i$ (absolute fluorescence) and motility parameters. **a:** hyperactivation (HA); **b:** curvilinear velocity (VCL; $\mu m.s^{-1}$); **c:** amplitude of lateral head displacement (ALH; μm); **d:** linearity (LIN; %). In all panels circles indicate pH_o=7.4 and triangles indicate pH_o=8.5. Yellow symbols show stimulation with P4 (controls green) and grey symbols show stimulation with 4-AP (controls black). Each point shows mean ± sem. For P4 experiments n=8 (fluorescence) and n=21 (motility parameters). For 4-AP experiments n=6 (fluorescence) and n=13 (motility parameters). R^2 values refer to line of best fit. In panels b-d the red dotted lines indicate threshold values for hyperactivation (Mortimer, 2000).

Figure 5.

Differing effects of P4 and 4-AP on straightness. **a**: Relationship between $[Ca^{2+}]_i$ (absolute fluorescence) and straightness (STR; %). Circles indicate pH_o=7.4 and triangles indicate pH_o=8.5; Yellow symbols show stimulation with P4 (controls green) and grey symbols show stimulation with 4-AP (controls black). Each point shows mean ± sem. For P4 experiments n=8 (fluorescence) and n=21 (STR). For 4-AP experiments n=6 (fluorescence) and n=13 (STR). **b:** Example of sperm track (black; points show position of sperm head in successive video frames). Curvilinear path (CL), average path (AP) and straight line path (SL) are shown by the black, red and, blue lines respectively. Respective velocities (VCL, VAP and VSL) are calculated by dividing each path length by time. Linearity (LIN) is calculated from the ratio between VSL and VCL and straightness (STR) is calculated from the ratio between VSL and VAP. **c:** Dose dependence of the effect of P4 on straightness (STR; %) at 300 s post-stimulus at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars). Mean ± sem of 14 experiments. **d:** Dose dependence of the effect of 4-AP on straightness (STR; %) at 300 s post-stimulus at pH_o=7.4 (grey bars) and pH_o=8.5 (black bars). Mean ± sem of 13 experiments. Asterisks indicates significant difference compared to untreated control; P<0.05 (*), P<0.01 (**), P<0.005 (***), P<0.001 (****).

Supplementary Information figure legends

Figure. S1.

Effects of vehicle (DMSO). **a:** Effect on fluo4 fluorescence of maximum dose of P4 (20 μM; blue trace) and equivalent concentration of vehicle (0.2% DMSO; black trace). Arrow shows time of P4/DMSO addition. Each trace shows mean of 8 experiments. **b:** Effect on fluo4 fluorescence of maximum dose of 4-AP (5 mM; blue trace) and equivalent concentration of vehicle (1% DMSO; black trace). Arrow shows time of 4-AP/DMSO addition. Each trace shows mean of 6 experiments. At lower doses (0.00001-0.1% DMSO) no effects were detected. **c-f:** 1% DMSO does not stimulate hyperactivation (c), or modify curvilinear velocity (VCL; d), amplitude of lateral head displacement (ALH; e) or linearity (LIN; f). Effects of 4-AP and P4 (assessed in parallel) are shown for comparison. Bars show mean (±SEM) of 6 experiments. P<0.05 (*), P<0.005 (***), P<0.001 (****).

Figure S2.

Determination of pH_i using BCECF. **a:** Assessment and calibration of pH_i. Seven aliquots of BCECF-loaded sperm were suspended in a multi-well plate, each at a different pH (indicated by different colour traces). Plot shows ratio of 490/440 emission in response to excitation at 530 nm. After recording for 60 s, 0.12% Triton X-100 was added simultaneously to each well (arrow) to permeabilise the cells, allowing equilibration of pH_i and pH_o. **b:** Calibration curve for relationship between BCECF emission ratio and pH_i. Mean ± sem of 5 separate experiments (as illustrated in panel 'a'). Fitted curve is polynomial ($y=0.5x^2+8.8x-33$). **c:** Effect of pH_o, over the range 6.0 to 9.0, on pH_i. Each point shows mean ± sem of 5 experiments

Figure S3.

Assessment of fluo4 saturation by the P4-induced $[Ca^{2+}]_i$ transient. **a:** Responses of fluo4-loaded sperm suspensions stimulated with P4 and ionomycin. Blue, green and red traces show effects of stimulation with 1 nM, 10 nM and 20 μM P4 (respectively) added at first arrow. In each case 10 μM ionomycin was added at the 2nd arrow. Black trace shows control with additions of vehicle only (0.2% DMSO, then 0.1% DMSO). **b:** Increment in fluo4 fluorescence (above pre-stimulus control) induced by 1 nM-20 μM P4 or 0.2% DMSO (black bars) and by subsequent addition of 10 μM ionomycin (blue bars). Cells were suspended in medium buffered to pH 8.5. Each bar shows mean ± sem of 6 experiments. **c:** Responses of sperm loaded with fluo5F to stimulation with 1 μM P4 (shown by arrow) at pH_o=7.4 (grey trace) and pH_o=8.5 (black trace). Each trace shows mean of 4 separate experiments from 4 different samples. **d:** Amplitude of $[Ca^{2+}]_i$ transient in fluo5F-loaded cells stimulated with 0.1 and 1 μM P4. Grey bars show pH_o=7.4, black bars show pH_o=8.5; mean ± sem of 4 experiments. Asterisks indicate comparison of responses to 0.1 μM and 1.0 μM P4 at pH_o=7.4 and pH_o=8.5. P<0.005 (***), P<0.001 (****).

Figure S4.

Effects of 4-AP and NH_4Cl on pH_i and hyperactivated motility. **a:** pH_i of sperm populations exposed to vehicle (0.4% DMSO), 2 mM 4-AP or 25 mM NH_4Cl for 300 s at pH_o=7.4 (grey) or pH_o=8.5 (black). Each bar shows mean ± sem of 5 experiments. * indicates P<0.05 compared to pH_i in DMSO controls and in cells exposed to 2 mM 4-AP. **b:** Hyperactivation (% cells) in sperm populations exposed to vehicle (0.4% DMSO), 2 mM 4-AP, or 25 mM NH_4Cl for 300 s at pH_o=7.4 (grey) or pH_o=8.5 (black). Each bar shows mean ± sem of 9 experiments. * and *** indicate P<0.05 and P<0.005 (respectively) compared to cells exposed to NH_4Cl in parallel experiments.

Figure 5.

An estimation of $[Ca^{2+}]_i$ was made by using amplitude of the transient response to P4 as a bioassay. (Harper et al., 2003), studied responses to P4 in populations of fura2-labeled human sperm which were prepared and capacitated using the same method as used here. In their study cells suspended at pH 7.4 (at 37^{o}C, not 30^{o}C as used here) were stimulated with P4 doses up to 30 μM and responses were calibrated using a standard ratiometric protocol for fura2. Using our data for responses to equivalent P4 stimuli (applied at pH_o=7.4), we constructed a plot relating absolute fluorescence of fluo4 at the transient peak to $[Ca^{2+}]_i$ (as assessed by fura2). Labels by each point show the dose of P4 applied (response saturates at 100 nM-1 μM). A linear regression was fitted to the points (R^2=0.97) and used to estimate $[Ca^{2+}]_i$ from mean fluorescence values.

Figure 1

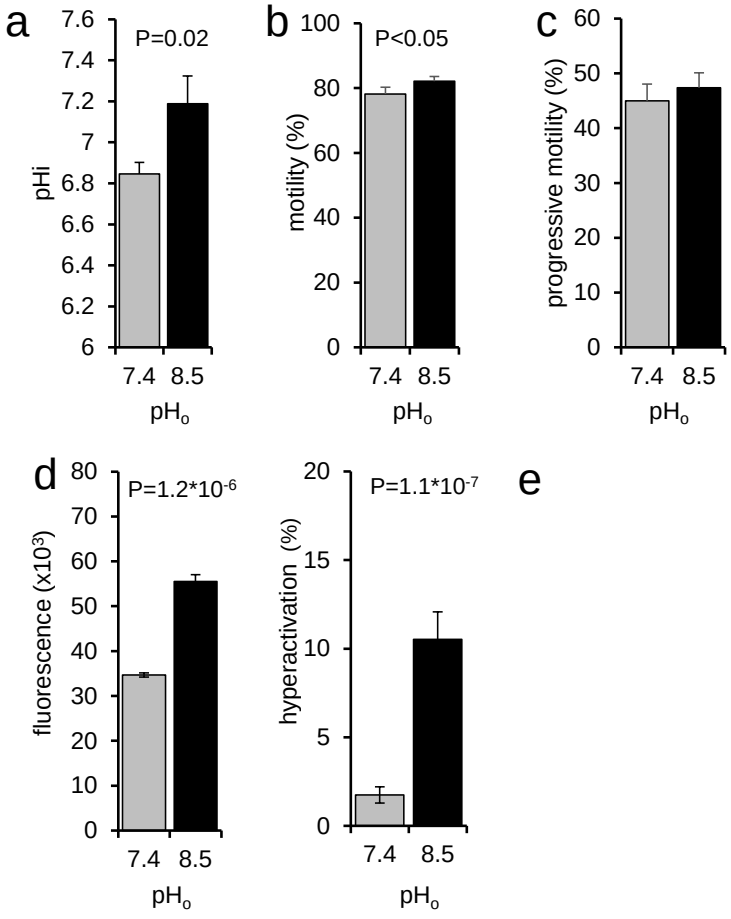

a
P=0.02
pHi
7.6
7.4
7.2
7
6.8
6.6
6.4
6.2
6
7.4 8.5
pHo
b
P<0.05
motility (%)
100
80
60
40
20
0
7.4 8.5
pHo
c
progressive motility (%)
60
50
40
30
20
10
0
7.4 8.5
pHo
d
P=1.2*10-6
fluorescence (x10³)
80
70
60
50
40
30
20
10
0
7.4 8.5
pHo
P=1.1*10-7
hyperactivation (%)
20
15
10
5
0
7.4 8.5
pHo
e

Figure 2

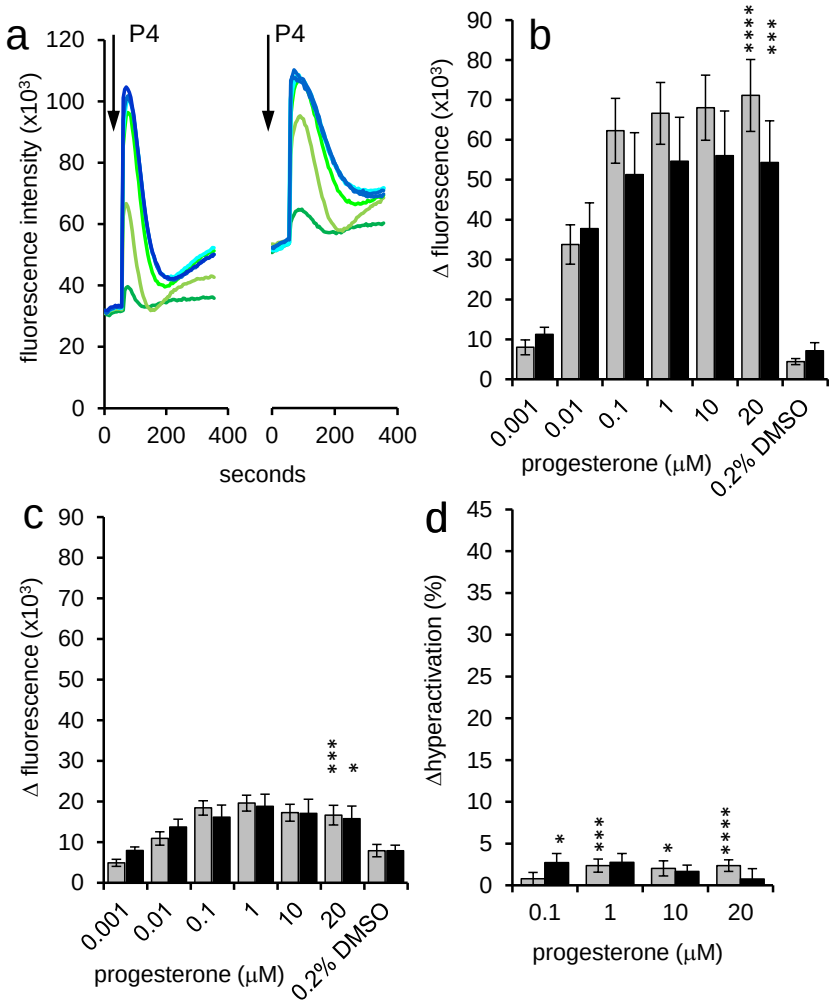
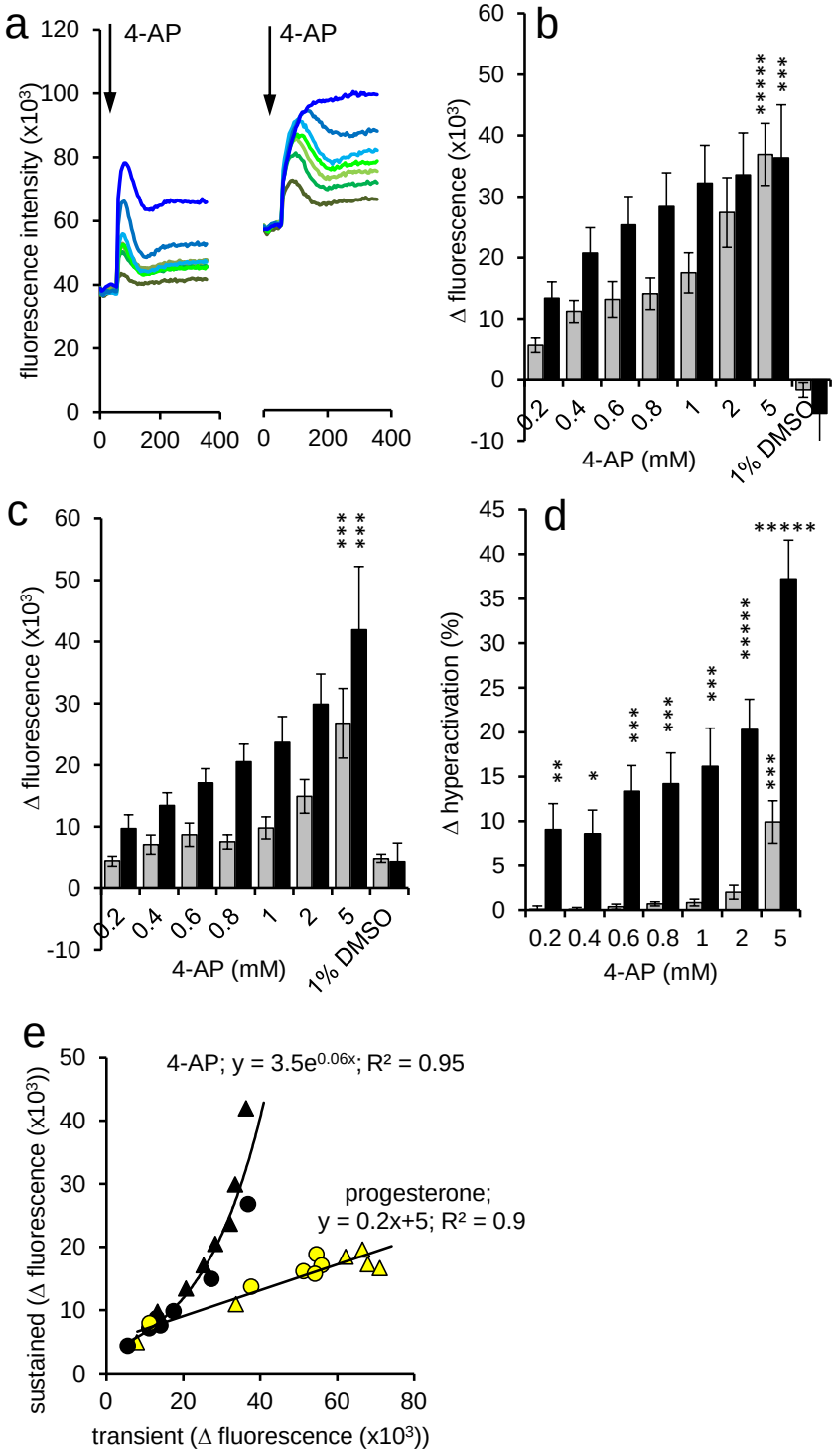
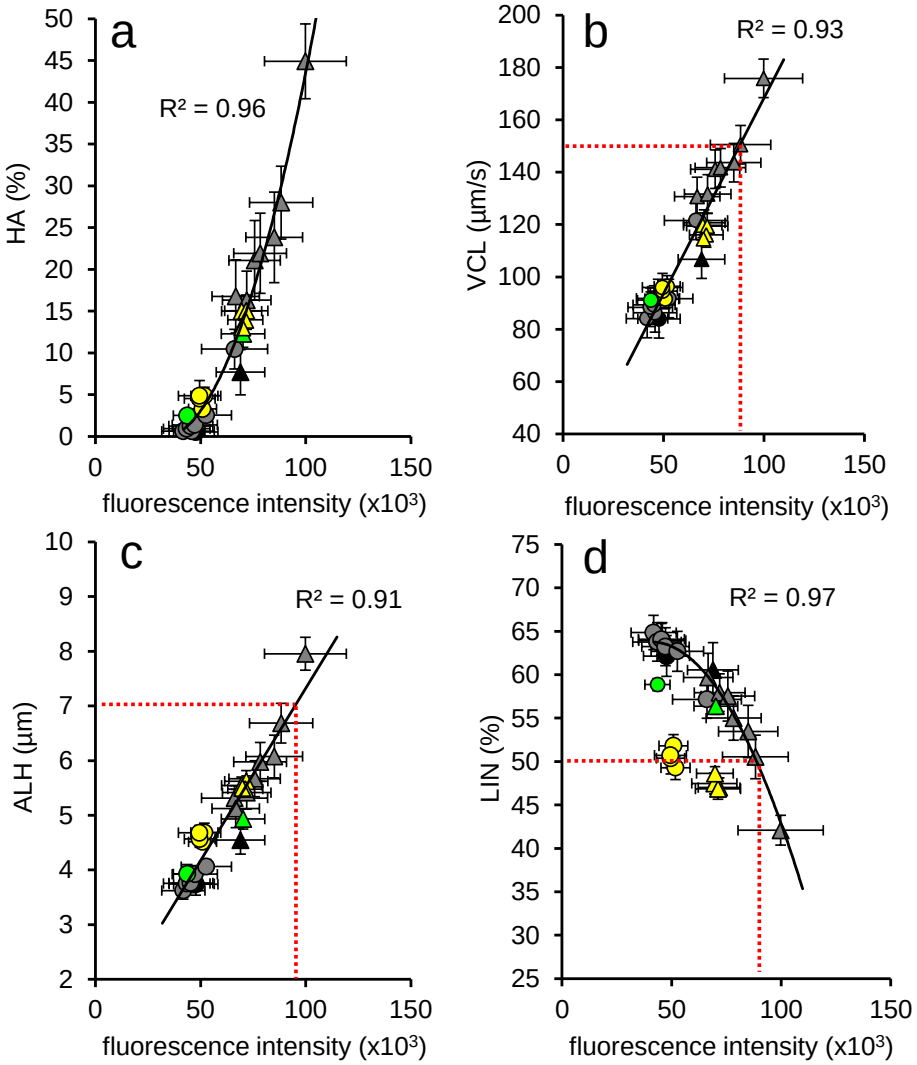

a
P4
P4
fluorescence intensity (x10³)
seconds
b
Δ fluorescence (x10³)
progesterone (μM)
0.2% DMSO
c
Δ fluorescence (x10³)
progesterone (μM)
0.2% DMSO
d
Δhyperactivation (%)
progesterone (μM)

Figure 3


a
4-AP
4-AP
fluorescence intensity (x10³)
b
Δ fluorescence (x10³)
4-AP (mM)
1% DMSO
c
Δ fluorescence (x10³)
4-AP (mM)
1% DMSO
d
Δ hyperactivation (%)
4-AP (mM)
e
4-AP; y = 3.5e^0.06x; R² = 0.95
progesterone;
y = 0.2x+5; R² = 0.9
sustained (Δ fluorescence (x10³))
transient (Δ fluorescence (x10³))

560

Figure 4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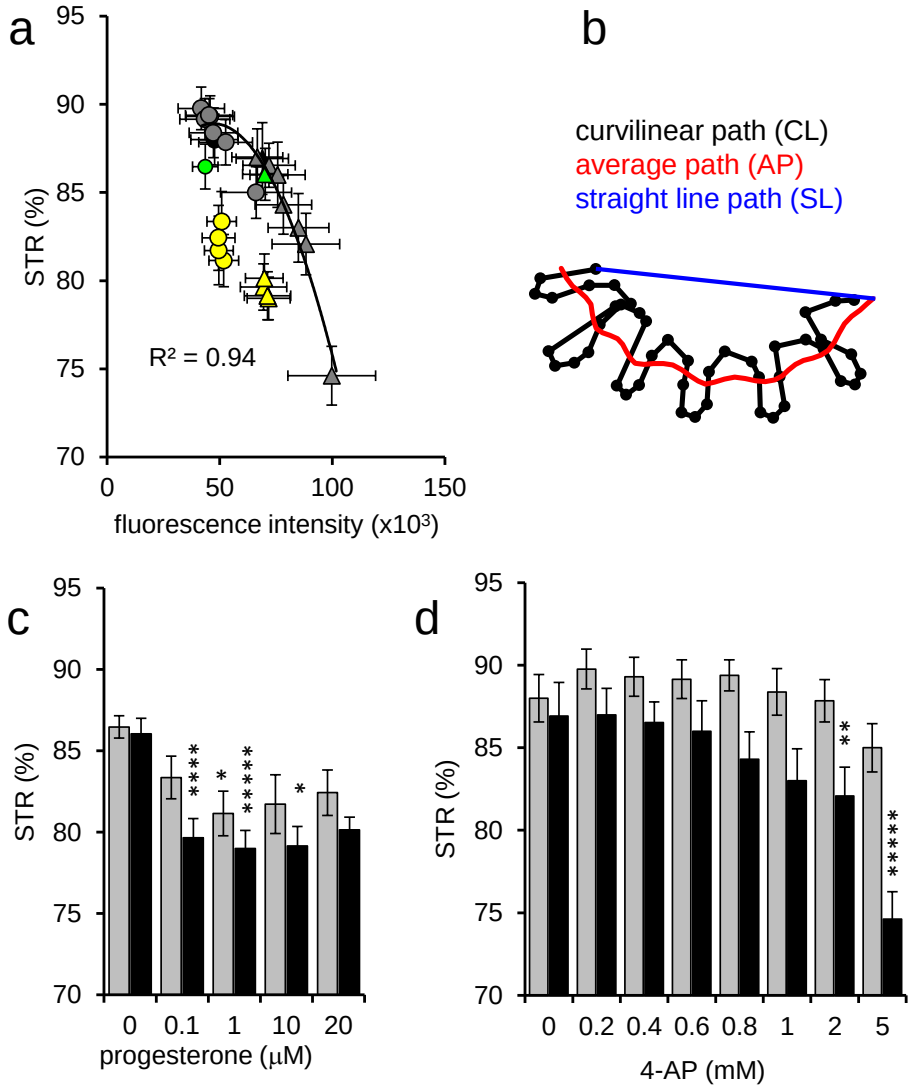
R² = 0.96
HA (%)
fluorescence intensity (x10³)
b
R² = 0.93
VCL (µm/s)
fluorescence intensity (x10³)
c
R² = 0.91
ALH (µm)
fluorescence intensity (x10³)
d
R² = 0.97
LIN (%)
fluorescence intensity (x10³)

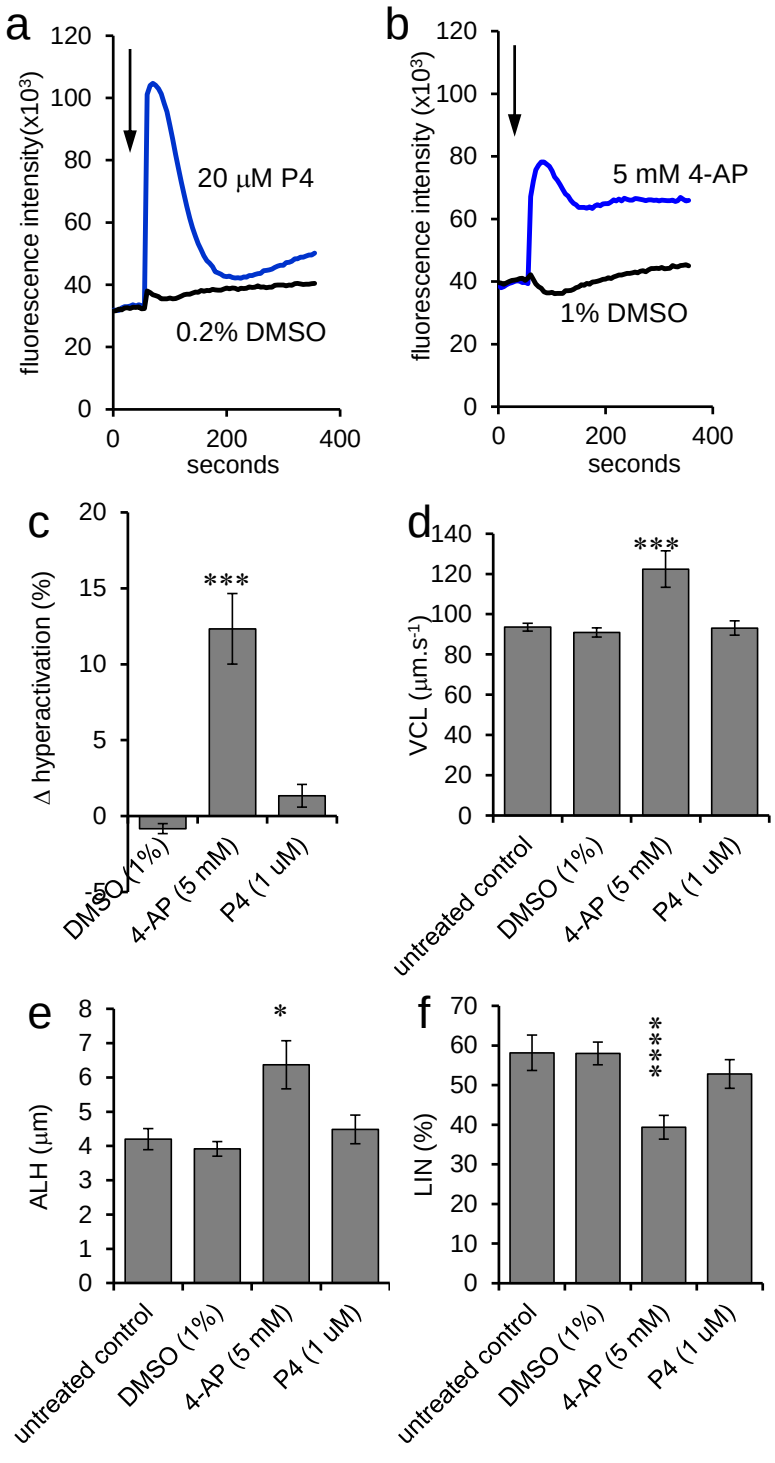
561

Figure 5


a
95
90
85
80
75
70
STR (%)
R² = 0.94
0
50
100
150
fluorescence intensity (x10³)
b
curvilinear path (CL)
average path (AP)
straight line path (SL)
c
95
90
85
80
75
70
STR (%)
****
*
****
*
0
0.1
1
10
20
progesterone (μM)
d
95
90
85
80
75
70
STR (%)
**
******
0
0.2
0.4
0.6
0.8
1
2
5
4-AP (mM)

562

Suppl. figure 1



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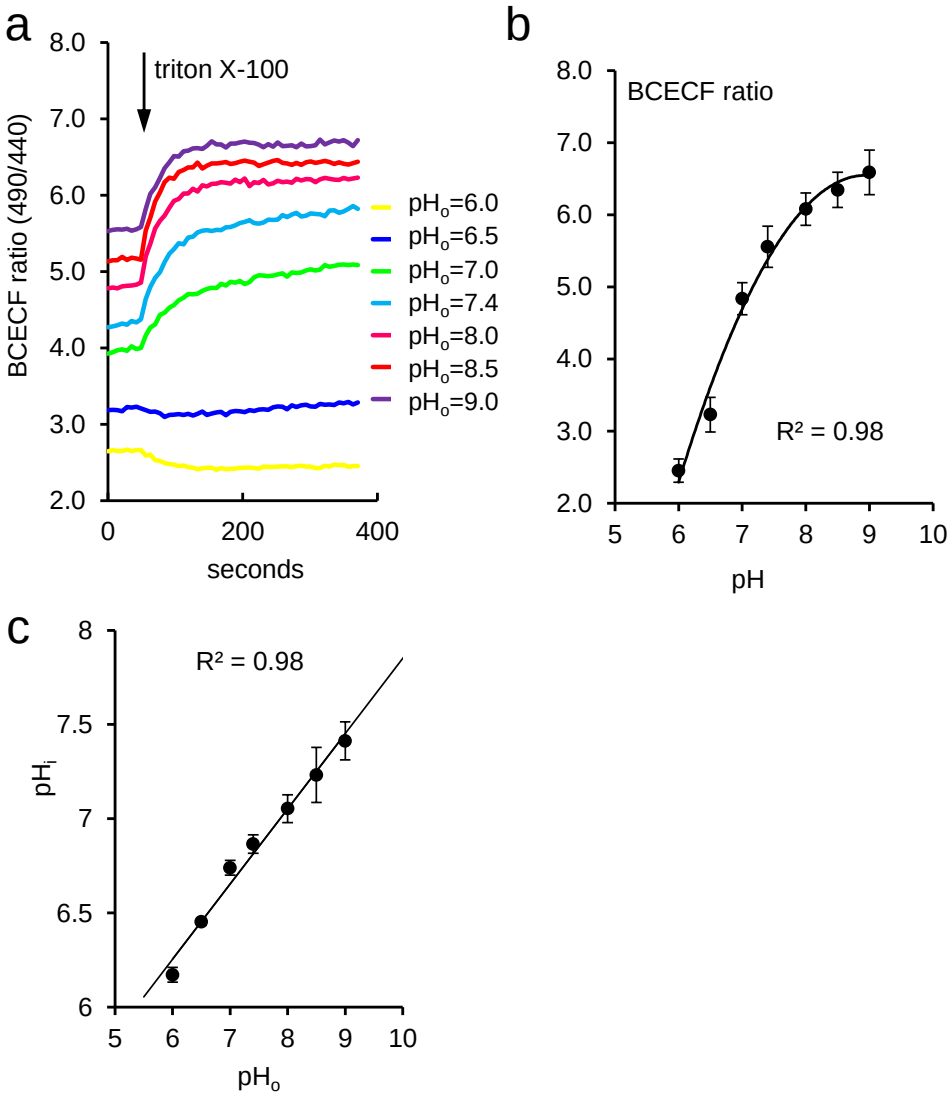
Suppl. figure 2



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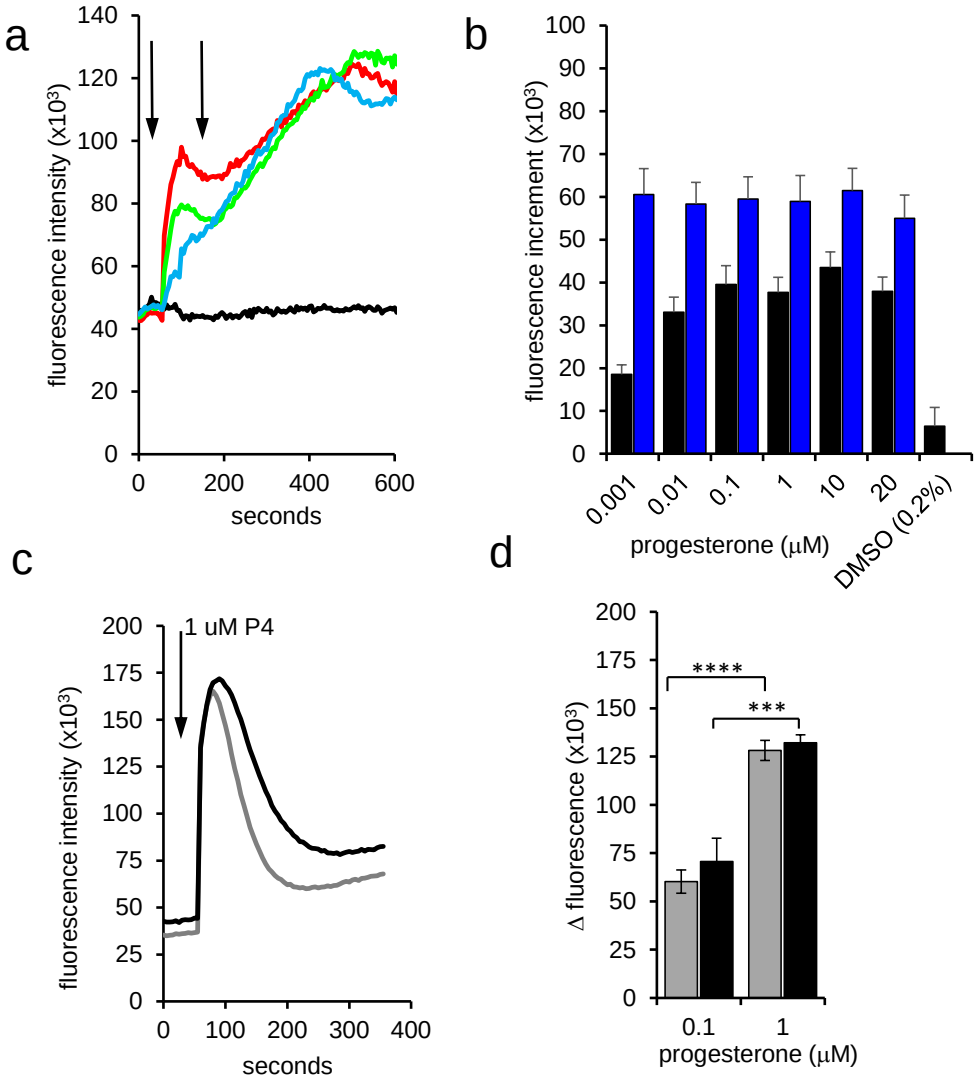
Suppl. figure 3



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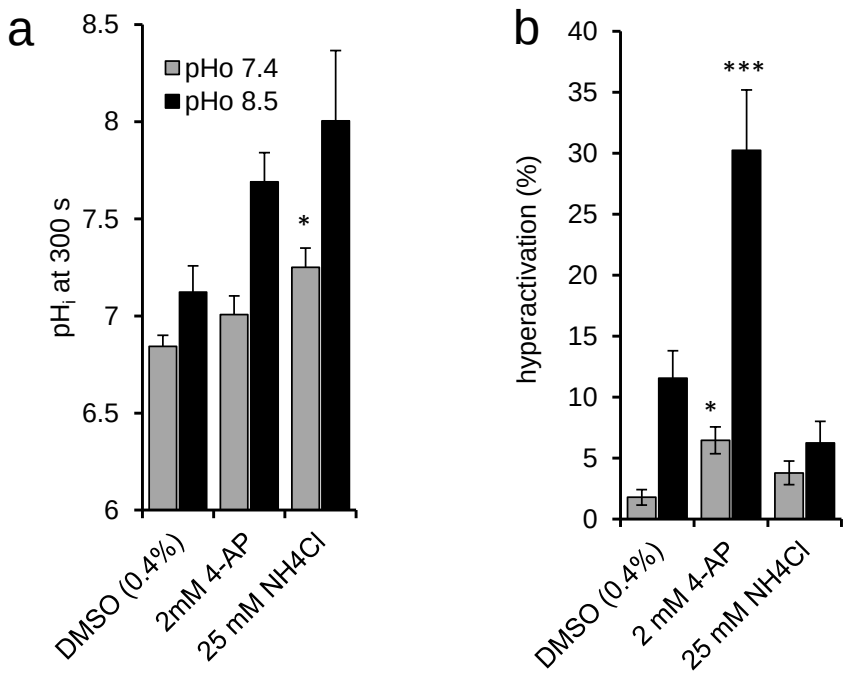
566

567

Suppl. figure 4



Suppl. figure 5